

Biochemical diagnosis of organophosphate-insensitivity with neural acetylcholinesterase extracted by sonication from the adult tick synganglion

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Received 27 October 2004; received in revised form 5 August 2005; accepted 7 September 2005

Abstract

A sonication method for the homogeneous extraction of acetylcholinesterase (AChE) from the synganglia of adult ticks is described. The method provides for the extraction of sufficient AChE for multiple assays of enzyme activity in the presence of specific organophosphate (OP) inhibitors for the rapid diagnosis of OP-insensitivity and assignment of homozygous susceptible (SS), heterozygous resistant (RS), and homozygous resistant (RR) genotypes to individual ticks. A single synganglion from adult ticks of either gender and various stages of feeding can successfully be used for AChE extraction. The study presents the results of diagnostic screening of four *Boophilus microplus* strains for OP-insensitivity. The extraction method and these findings should find utility in support of researchers involved in the mitigation of acaricide resistance in tick populations worldwide, and in particular, the Cattle Fever Tick Surveillance and Quarantine Program maintained by USDA-APHIS/Veterinary Services along the south Texas border that prevents reentry of *Boophilus* spp. into the United States from endemic populations in Mexico. © 2005 Elsevier B.V. All rights reserved.

Keywords: Acaricide resistance; Acetylcholinesterase; Organophosphate insensitivity

1. Introduction

The Cattle Fever tick, *Boophilus annulatus* (Say) and the southern cattle tick, *B. microplus* (Canestrini) are the arthropod vectors of *Babesia bovis* and *B. bigemina*, the protozoan parasites that cause bovine babesiosis (Smith and Kilborne, 1893). To eliminate this disease from southern cattle herds, these ticks

were eradicated from the United States in 1943 through a program of pasture vacation and arsenical acaricides (Graham and Hourigan, 1977). Of course, both *Boophilus* spp. remain endemic in Mexico and are a constant threat for reintroduction into the United States. *B. microplus* is generally considered the greater threat for reintroduction because of host adaptability and suspected greater genetic vigor (Graham and Hourigan, 1977). A vigilant surveillance and quarantine program is maintained by the USDA-APHIS/Veterinary Services along the Texas–Mexico border

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from Del Rio, TX to Brownsville, TX to prevent reentry of the tick into the United States (George, 1996). Cattle entering the United States from Mexico are presented for importation at designated ports as tick free. These previously treated cattle are palpated and observed visually for ticks by program inspectors. If they are found free of ticks, they are supplementary dipped in vats charged with the organophosphate (OP) acaricide coumaphos, and certified for entry into the United States (George, 1996).

Acaricide treatment is the vanguard defense against reentry of the tick into the United States. Continued effectiveness of the quarantine program is dependent upon the efficacy of the acaricides that are available for use, particularly the OP coumaphos (George, 1996). A limited number of existing acaricides are available for vat formulation. In addition, with such a limited market, it is unlikely that industry would have the economic incentive to develop new compounds for that specific use. The development of acaricide resistance within the *B. microplus* population would greatly hamper the continued success of the program. Thus, with reports of OP and pyrethroid resistance in Mexico (Santamaria and Fragoso, 1994; Fragoso et al., 1995), concerns are raised about the eventuality of acaricide resistant ticks entering the United States. These concerns provide the impetus for the development of diagnostic tools that can be used by the program for management of OP resistance development within the Mexican *B. microplus* population.

Currently, diagnostic bioassay for resistance to coumaphos is dependent upon collection of females that are sufficiently engorged to allow oviposition and serve as parental types for the strain. When larvae are in sufficient number, they are bioassayed by the larval packet test (George, 1996). Obviously, this process is laborious and can take several weeks to conduct. A more rapid, single tick assay would greatly facilitate diagnosis and provide a superior resistance management tool.

AChE OP-insensitivity is defined by a slower rate of AChE inhibition in the resistant phenotype, and those individuals possessing this trait are referred to as OP-resistant. AChE OP-insensitivity to dioxathion, carbophenothion, and formothion was demonstrated to be the result of an apparent single autosomal gene that is incompletely dominant with

respect to the susceptible allele (Stone, 1968a), and as such the homozygous susceptible, heterozygous resistant, and homozygous resistant larvae from defined adult pairings could be segregated into these phenotypes/genotypes by bioassay LD₅₀ results. In response to a need for a rapid, qualitative assay for the management of OP resistance in Australia and worldwide, Baxter et al. (1999) developed a single tick assay based upon AChE extracted from the synganglion, or “brain”, of the engorged adult female tick and inhibited in the presence of the carbamate propoxur. This assay can be used to diagnose OP-insensitivity and segregate the results into susceptible and resistant genotypes.

Low AChE activity is a characteristic of the resistant phenotype (Lee and Bantham, 1966). Thus, low AChE activity caused by incomplete and less than homogenous extraction of AChE from the synganglion can lead to the mistaken classification of the individual as OP-resistant. The purpose of the current study is to improve upon the method of AChE extraction from the synganglia of adult male and female ticks, providing homogenous extractions between samples, and increased amounts of AChE for quantitative biochemical assays.

2. Materials and methods

2.1. Tick strains and life stages

The synganglia of *Amblyomma americanum* adults were used to develop the sonication method of AChE extraction. The *A. americanum* colony is maintained at the Knipping-Bushland U.S. Livestock Insects Laboratory in Kerrville, TX. To evaluate the technique for its diagnostic potential, four *Boophilus microplus* tick strains, one susceptible to OP (Munoz, F₂₄), two resistant to OP (San Roman, F₃₄ and Pesqueria, F₁₉), and an outbreak strain (Arce-2, F₃) were used. The Munoz and San Roman strains have been described in a previous publication (Pruett, 2002). The Pesqueria strain was described by Li et al. (2003) as a strain collected at the U.S. port-of-entry in Reynosa, Tamaulipas, Mexico, by the USDA Veterinary Service inspectors from cattle originating from Pesqueria, Nuevo Leon, Mexico. The Arce pasture II strain was obtained from an

outbreak within the quarantine zone, in Starr County, TX. This was the second submission of ticks from this location after the cattle had been treated by spray-dipped with 0.25% AI coumaphos (Dr. Ronald Davey, CFTRL, personal communication). These tick strains are maintained in quarantine at the Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX.

Both *A. americanum* and *B. microplus* ticks used in the study were fed on, and collected, from cattle. Life stages of *A. americanum* include unfed male and female ticks and ticks collected from cattle on the host for 5 days (female holding stage unmated). The *B. microplus*, male, and not fully engorged female adults, were collected 18 days after larvae were placed on the calf.

2.2. Standard collection of adult tick synganglion for comparison of grinding versus sonication

The adult tick synganglion (male and female) was removed by dissection and placed into a 1.5 ml microcentrifuge tube containing 100 μ l of tick extraction buffer (TEB: 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, and 0.5% Triton X-100). Those synganglia disrupted by grinding were ground in 1.5 ml centrifuge tubes with plastic pestles (PGC Scientifics, Frederick, MD). AChE was extracted for 1 h at 4 °C. Extracted AChE was harvested in the supernatant following centrifugation at $14,400 \times g$ for 15 min. Sonication of the synganglion was accomplished with a Vibra Cell High Intensity Ultrasonic Processor (Model VC60, Danbury, CT) using a 2 mm microtip probe with power set at 7–9 W delivered to the TEB. The optimum sonication time was determined by experimentation (data not shown) and found to be 4 cycles of 3 s bursts with a 5 s interval between bursts. As above AChE was extracted for 1 h at 4 °C and harvested by centrifugation.

2.3. Standard AChE assay

AChE activity of synganglion extracts was measured with a modification of the Ellman assay (Ellman et al., 1961). A microplate method was developed that utilized acetylthiocholine iodide (ASCh) as substrate. The substrate solution (0.12 mM) was prepared in

50 mM sodium phosphate buffer, pH 7.5, containing 0.32 mM Ellman's reagent, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB, Sigma Chemical Co., St. Louis, MO). The standard assay for AChE activity consisted of 20 μ l of AChE extract and 180 μ l of substrate solution. The reaction rate was monitored at 30 °C for 1 h, measuring the absorbance at 405 nm at 10 min intervals, with a 5 s shake prior to reading using a Bio-Tek EL808 plate reader (Bio-Tek Instruments Inc., Winooski, VT). AChE activity is presented as the slope of the reaction rate created by the increase in absorbance over time. Analysis of data distributed normally (*B. microplus*) was accomplished with a One Way analysis of variance (ANOVA) and all pair-wise comparisons were made with the Tukey test. If the data failed a test of normality (*A. americanum*), they were analyzed with a Kruskal–Wallis ANOVA on ranks and all pair-wise comparisons made by the Dunn's method ($P < 0.05$, SigmaStat, SYSTAT Software Inc., Chicago, IL).

2.4. Inhibition of AChE activity of *B. microplus* strains by paraoxon, coroxon, and diazoxon

The oxon forms of parathion, coumaphos, and diazinon at a dilution of 2.5×10^{-5} M, were used in OP-inhibition experiments. In this experiment, an individual synganglion from partially engorged *B. microplus* females, of four different strains, was extracted in 125 μ l of TEB by sonication. Extracted AChE (25 μ l) from an individual synganglion was added to the microtiter plate well. The appropriate OP-inhibitor (paraoxon, coroxon, or diazoxon 2.5×10^{-5} M) was added to the AChE in an equal volume (25 μ l) and allowed to incubate with the AChE for 10 min at room temperature. Following the incubation period 180 μ l of substrate (as in the standard AChE assay, 0.12 mM ASCh) was added to the well and the reaction monitored as stated above in the standard AChE assay. The uninhibited AChE reaction was treated the same as the inhibited reactions except that 25 μ l of 50 mM sodium phosphate buffer, pH 7.5, was added to the AChE sample without inhibitor. The effect of inhibition was expressed as a percentage (%) of AChE activity remaining at the 30 min timed reading $[(\Delta \text{ absorbance of the inhibited reaction} / \Delta \text{ absorbance of the uninhibited reaction}) \times 100]$.

3. Results

3.1. Comparison of AChE extraction by grinding and sonication

Significantly greater reaction slopes ($P < 0.05$) indicate more AChE activity per volume was extracted from the synganglion of *A. americanum* and *B. microplus* ticks by sonication than by grinding of the synganglion with a pestle (Table 1). There was no difference in extractable AChE between genders using either sonication or grinding.

Twenty synganglia were collected from individual females from the control susceptible Munoz strain of *B. microplus*. AChE was extracted from each individual synganglion by grinding ($n = 10$) or by sonication ($n = 10$). The mean change in optical density over 30 min for AChE activity extracted by grinding was 0.038 OD with a standard deviation of 0.026, while the value for AChE activity extracted by sonication was 1.8-fold greater at 0.067 OD with a lower standard deviation of 0.023 (Table 2).

3.2. Segregation of biochemical data into genotypes

Table 3 contains the combined biochemical results obtained from AChE extracted from sonicated synganglia of the four different *B. microplus* strains. Values for % AChE activity remaining after 30 min in

the presence of specific OP-inhibitors can be segregated into three groups representing the three different genotypes, homozygous susceptible (SS), heterozygous (RS), and homozygous resistant (RR). In the presence of paraoxon the SS genotype retained a mean of 26.4% (± 7.3 , $n = 14$) activity relative to the control uninhibited reaction at 30 min, the RS genotype 58.8% (± 5.9 , $n = 13$) and the RR genotype 94.8% (± 5.4 , $n = 13$). These values were all significantly different from each other ($P < 0.05$). The results for the OP-inhibitors coroxon and diazoxon followed the same pattern as paraoxon (Table 3). At the inhibitor concentration evaluated toxicity followed the pattern of coroxon > paraoxon > diazoxon. The results indicate that in the resistant genotype diazoxon actually enhanced AChE activity as the RR genotype had a mean 121.3% (± 21.2 , $n = 14$) activity relative to the uninhibited control at 30 min.

Homozygous OP-resistant *B. microplus* ticks exhibited very low levels of uninhibited AChE activity, significantly lower than the uninhibited AChE activity of the homozygous susceptible and heterozygous ticks (Table 3). The loss of AChE activity in the paraoxon defined RR genotype was significant ($P < 0.05$) with a mean ΔOD of 0.011 (± 0.003 , $n = 13$) after 30 min of uninhibited reaction, a value representing only 14.3% of the SS genotype. This was also consistent with those ticks defined as RR genotypes with the OP-inhibitors coroxon and diazoxon. There was no significant difference between

Table 1

A comparison of AChE activity extracted from the synganglion of *Amblyomma americanum* (A. a.) and *Boophilus microplus* (B. m.) by either grinding or sonication

Species	Gender	Status	<i>n</i>	Treatment	Slope (mean)	S.D.	Slope (median)	$P < 0.05$
A. a.	Female	Unfed	20	Grind	0.922	0.423	0.997	a
A. a.	Female	Unfed	20	Sonicated	1.837	0.653	1.601	b
A. a.	Female	Holding	20	Grind	0.717	0.400	0.726	a
A. a.	Female	Holding	20	Sonicated	1.879	0.628	1.764	b
A. a.	Male	Unfed	20	Grind	0.762	0.367	0.620	a
A. a.	Male	Unfed	20	Sonicated	1.618	0.511	1.764	b
B. m.	Female	Fed	20	Grind	0.892	0.462	0.721	a
B. m.	Female	Fed	20	Sonicated	3.095	0.844	2.934	b
B. m.	Male	Fed	20	Grind	1.108	0.629	1.010	a
B. m.	Male	Fed	20	Sonicated	3.632	0.898	3.571	b

AChE activity is expressed as the slope of the reaction rate (increase in absorbance over time). Results of *A. americanum* and *B. microplus* were not compared as both species were analyzed for significant differences independently. The *A. americanum* results were analyzed with the Kruskal–Wallis ANOVA on ranks ($H = 74.702$, d.f. = 5, $P < 0.001$), and the *B. microplus* results were analyzed with a One Way ANOVA ($F = 72.08$, d.f. = 79, $P < 0.001$). Different letters in the $P < 0.05$ column indicate significant differences between pair-wise comparisons.

Table 2

Two extraction methods, grinding and sonication, were compared for the homogenous recovery of AChE from the synganglion of adult *B. microplus* female ticks (Munoz strain)

Sample	Extraction method	ΔOD NI ^a	ΔOD I ^b	% Activity ^c	Genotype
1	Grind	0.010	0.008	80.0	RR
2	Grind	0.056	0.017	30.4	SS
3	Grind	0.023	0.011	47.8	RS
4	Grind	0.035	0.012	34.3	SS
5	Grind	0.025	0.013	52.0	RS
6	Grind	0.067	0.016	23.9	SS
7	Grind	0.026	0.009	34.6	SS
8	Grind	0.021	0.009	42.9	SS
9	Grind	0.027	0.008	29.6	SS
10	Grind	0.094	0.041	43.6	SS
Mean \pm S.D.		0.038 \pm 0.026	0.014 \pm 0.010	41.9 \pm 16.0	
1	Sonicated	0.088	0.025	28.4	SS
2	Sonicated	0.072	0.018	25.0	SS
3	Sonicated	0.039	0.022	56.4	RS
4	Sonicated	0.080	0.020	25.0	SS
5	Sonicated	0.090	0.019	21.1	SS
6	Sonicated	0.095	0.014	14.7	SS
7	Sonicated	0.067	0.012	17.9	SS
8	Sonicated	0.074	0.015	20.3	SS
9	Sonicated	0.031	0.017	54.8	RS
10	Sonicated	0.038	0.012	31.6	SS
Mean \pm S.D.		0.067 \pm 0.023	0.017 \pm 0.004	29.5 \pm 14.6	

Sonication yielded a significantly higher AChE reaction rate (ΔOD NI) than did grinding (*t*-test, *t* = -2.628, d.f. = 18, *P* = 0.017). Genotype determined by grouping the results of % activity remaining after 30 min of exposure to OP into homozygous susceptible (SS), heterozygous resistant (RS), and homozygous resistant (RR) genotypes. Susceptible allele denoted by S and resistant allele denoted by R.

^a Change in optical density after 30 min in the control AChE + ASCh reaction with no inhibitor (NI).

^b Change in optical density after 30 min in the AChE + ASCh reaction with specific inhibitor (I = paraoxon).

^c Percent of AChE activity remaining in the inhibited reaction relative to the uninhibited control reaction after 30 min.

the paraoxon, coroxon, and diazoxon defined SS and RS genotypes relative to the AChE reaction rate (ΔOD) after 30 min of the uninhibited reaction.

3.3. Diagnostic screening of four *B. microplus* strains for OP-insensitivity

Females from four *B. microplus* strains were screened for OP-insensitivity with the AChE inhibition assay using AChE extracted by sonication, as described in the methods. The strains included a control susceptible strain (Munoz), two OP-resistant strains (San Roman and Pesqueria), and an outbreak strain (Arce-2). In the presence of paraoxon, coroxon, and diazoxon, the OP susceptible Munoz strain had no homozygous (RR) genotypes and a high frequency of the susceptible allele, including 8 of 10 individuals found to be homozygous susceptible (Table 4).

However, the resistant allele was observed in the susceptible strain as two heterozygotes were identified for each OP-inhibitor tested. A high frequency distribution of the resistant allele, either of the RS or RR genotype, was predictably observed in the OP-resistant strains. In the presence of paraoxon and coroxon only one homozygous susceptible individual was found in the San Roman strain, and none in the Pesqueria strain. Interestingly the outbreak strain had a somewhat even distribution of homozygous susceptible and homozygous resistant individuals (SS:RS:RR) at 5:1:4 for paraoxon and coroxon. Unlike paraoxon and coroxon, the OP-inhibitor diazoxon yielded a much different genotype ratio for the Arce-2, San Roman, and Pesqueria strains with a fairly even distribution between homozygous susceptible and homozygous resistant genotypes with only two heterozygotes (Table 4).

Table 3

AChE assay results of individual *B. microplus* females of the Munoz, Arce-2, San Roman, and Pesqueria strains combined and segregated into OP-susceptible (SS), OP-resistant heterozygote (RS) and OP-resistant homozygote (RR) genotypes based upon AChE activity in the presence of specific OP inhibitors

Paraoxon			
Genotype	SS (<i>n</i> = 14)	RS (<i>n</i> = 13)	RR (<i>n</i> = 13)
ΔOD 30 min ^a	0.077 (0.039) a	0.071 (0.048) a	0.011 (0.003) b
% Activity ^b	26.4 (7.3) a	58.8 (5.9) b	94.8 (5.4) c
Coroxon			
Genotype	SS (<i>n</i> = 14)	RS (<i>n</i> = 13)	RR (<i>n</i> = 13)
ΔOD 30 min	0.077 (0.039) a	0.067 (0.051) a	0.016 (0.018) b
% Activity	23.5 (5.7) a	55.7 (6.3) b	80.2 (4.4) c
Diazoxon			
Genotype	SS (<i>n</i> = 21)	RS (<i>n</i> = 4)	RR (<i>n</i> = 14)
ΔOD 30 min	0.071 (0.045) a	0.083 (0.058) a	0.023 (0.024) b
% Activity	57.9 (13.2) a	92.4 (2.5) b	121.3 (21.2) c

Data analyzed by Kruskal–Wallis ANOVA on ranks for ΔOD 30 min, paraoxon ($H = 25.671$, d.f. = 2, $P < 0.001$), coroxon ($H = 21.611$, d.f. = 2, $P < 0.001$), and diazoxon ($H = 16.592$, d.f. = 2, $P < 0.001$). Data analyzed by One Way ANOVA for % activity, paraoxon ($F = 398.401$, d.f. = 39, $P < 0.001$), coroxon ($F = 361.033$, d.f. = 39, $P < 0.001$), and diazoxon ($F = 65.574$, d.f. = 38, $P < 0.001$). Significant differences between pair-wise comparisons (Dunn's method) in rows indicated by different letters (a, b, and c) $P < 0.05$.

^a The mean (±standard deviation) change in optical density after 30 min with the uninhibited control reaction (AChE + ASCh).

^b The mean (±standard deviation) percent of AChE activity remaining in the inhibited reaction relative to the uninhibited control reaction after 30 min.

4. Discussion

AChE is the metabolic target-site for OP and carbamate pesticides (Fournier and Mutero, 1994). Binding of OP to AChE quasi-irreversibly inhibits

enzyme activity and with sufficient AChE inhibition, death of the organism (O'Brien, 1967, p. 232). Smissaert et al. (1975) have determined, in OP-susceptible spider mites, that 10% is the minimal fraction of acetylcholinesterase compatible with life.

Table 4

Mean (±standard deviation) AChE assay results for specific *B. microplus* strains in the presence of OP-inhibitors

Strain	<i>n</i>	OP-Inhibitor	ΔOD NI ^a	ΔOD I ^b	% Activity ^c	SS:RS:RR ^d
Munoz	10	Paraoxon	0.067 (0.023)	0.017 (0.004)	29.5 (14.6)	8:2:0
Arce-2	10	Paraoxon	0.034 (0.023)	0.013 (0.004)	57.6 (33.8)	5:1:4
San Roman	10	Paraoxon	0.073 (0.065)	0.043 (0.031)	73.0 (18.6)	1:5:4
Pesqueria	10	Paraoxon	0.041 (0.052)	0.027 (0.034)	76.4 (21.0)	0:5:5
Munoz	10	Coroxon	0.067 (0.023)	0.016 (0.004)	27.3 (14.0)	8:2:0
Arce-2	10	Coroxon	0.034 (0.023)	0.011 (0.003)	48.5 (26.0)	5:1:4
San Roman	10	Coroxon	0.073 (0.065)	0.041 (0.030)	66.2 (15.0)	1:5:4
Pesqueria	10	Coroxon	0.041 (0.052)	0.024 (0.032)	67.6 (16.5)	0:5:5
Munoz	10	Diazoxon	0.067 (0.023)	0.039 (0.020)	59.6 (22.3)	8:2:0
Arce-2	10	Diazoxon	0.034 (0.023)	0.024 (0.011)	86.0 (33.8)	6:0:4
San Roman	10	Diazoxon	0.073 (0.065)	0.052 (0.041)	85.9 (25.2)	4:2:4
Pesqueria	9	Diazoxon	0.041 (0.052)	0.039 (0.051)	107.6 (38.7)	3:0:6

Each strain represented by AChE extracted by sonication from 10 individual female synganglia in 125 μl of extraction buffer and the extract portioned into 25 μl aliquots for exposure to the specific inhibitor.

^a Change in optical density after 30 min in the control AChE + ASCh reaction with no inhibitor (NI).

^b Change in optical density after 30 min in the AChE + ASCh reaction with specific inhibitor (I).

^c Percent of AChE activity remaining in the inhibited reaction relative to the uninhibited control reaction after 30 min.

^d Genotype ratio, susceptible (S) allele and resistant (R) allele.

The rate that OP inhibits AChE activity is dependent upon the affinity of OP for AChE, defined by the dissociation constant (K_d), and/or the rate of phosphorylation of the active-site serine, defined by the phosphorylation constant (k_2) (Hart and O'Brien, 1973; Wilkinson, 1976, p. 282).

AChE insensitivity is considered the principal resistance mechanism associated with OP resistance in *B. microplus* (Lee and Bantham, 1966). AChE insensitivity reflects a presumptive conformational alteration within an individual AChE that diminishes the inhibitory effect (rate of inhibition) of OP upon AChE activity (Smissaert, 1964). Pruett (2002) reported that the bimolecular reaction constants (k_i) for AChEs extracted from OP-resistant Caporal and San Roman *B. microplus* strains maintained at the CFTRL were most affected by a slower rate of enzyme phosphorylation (k_2).

Lee and Bantham (1966), and Stone (1968b) suggested that in the homozygous resistant *B. microplus* genotype the AChE insensitivity was coupled with a significant loss of AChE activity, approximately 12% of the susceptible genotype. Through a series of well-designed genetic crossing experiments between Australian *B. microplus* OP-resistant and -susceptible strains, it was determined that the OP-resistant phenotype was apparently inherited as a single autosomal gene incompletely dominant with respect to the susceptible allele (Stone, 1968b). Stone (1968b) suggested that low brain AChE activity and resistance to OP-inhibition are either pleiotropic effects of the same gene, or two closely linked genes that control these traits. These results would suggest that allelic differences, resulting from a mutation within the gene, would account for the lower brain AChE activity and resistance to OP-inhibition. Nolan et al. (1972) separated, by electrophoresis, five forms (bands) of AChE activity from resistant and susceptible strains of *B. microplus*. Two forms exhibited similar total AChE activity and sensitivity to the OP-inhibitor coroxon and were considered the susceptible component. The other three forms demonstrated lower AChE activity and were insensitive to coroxon inhibition. They considered these forms associated with the resistant component. In subsequent experiments, Nolan and Schnitzerling (1975) characterized and demonstrated that the altered AChE, which they referred to as the critical

component of resistance, had an optimum substrate (acetylthiocholine iodide) concentration of 1.73×10^{-2} M, while the susceptible component had an optimal substrate concentration of 2.7×10^{-4} M. This was also reflected in a reduced K_m for the resistant enzyme (6.43×10^{-6} M) relative to the susceptible enzyme (1.98×10^{-5} M), suggesting an increase in affinity of the resistant enzyme for the substrate with a correspondingly lower V_{max} . The changes reflected in the V_{max} and K_m of the resistant enzyme suggests structural changes that result from mutation or post-translational changes.

Although mutation within the *B. microplus* AChE gene has also been suggested through biochemical analysis (Wright and Ahrens, 1988; Pruett, 2002), evaluation of sequence information from three putative AChE cDNAs (AChE1, AChE2, and AChE3) has revealed no OP-insensitivity-associated mutations (Baxter and Barker, 1998; Hernandez et al., 1999; Temeyer et al., 2004). The absence of defined OP-insensitivity-associated mutations prevents the design of diagnostic molecular probes. However, studies by Stone et al. (1976) and Baxter et al. (1999) have demonstrated, with Australian *B. microplus* strains, that single tick genotypes for OP-resistance can be diagnosed by biochemical assay.

In the current study the results of AChE inhibition experiments upon AChE extracted from the synganglion of individual female ticks from Texas and Mexican strains of *B. microplus* were also segregated into three distinct groups, the apparent homozygous susceptible (SS), heterozygous (RS), and homozygous resistant (RR) genotypes (Table 3). We found no significant difference in uninhibited AChE activity between the SS and RS genotype. However, in agreement with the results of Stone (1968b) the uninhibited AChE activity of the RR genotype was 14.2% of the SS genotype, and 15.5% of the RS genotype.

Sonication of the synganglion significantly increased the amount of AChE extracted over disrupting the tissue by grinding (Tables 1 and 2). Sonication affords optimal homogenous extraction of AChE from the neural tissue of an adult individual, regardless of gender and feeding-stage (Table 1). Optimal extraction of AChE allows sufficient AChE for evaluation of OP-insensitivity for several OP class acaricides. At the inhibitor concentration evaluated in

the present study (2.5×10^{-5} M) toxicity followed the pattern of coroxon > paraoxon > diazoxon. Both coroxon and paraoxon are diethyl-OPs and they yielded similar results in terms of OP-insensitivity diagnosis. However, diazoxon also a diethyl-OP yielded quite different results for the RR genotype. It appeared that AChE activity was enhanced rather than inhibited in the presence of 2.5×10^{-5} M diazoxon yielding a mean % activity of 121.3% relative to the uninhibited control. Although beyond the scope of the present study, this observation deserves further investigation.

Currently the only rapid diagnostic alternative to bioassay is the biochemical assay of OP-insensitivity. Baxter et al. (1999) introduced a *B. microplus* AChE insensitivity assay with AChE extracted from the engorged female synganglion. The specific inhibitor used in the assay was propoxur, a carbamate, and a discriminating concentration of propoxur was used for the diagnosis of AChE insensitivity. Extraction of AChE was achieved by homogenizing (grinding with glass/Teflon) the synganglion in a small (10 μ l) volume of buffer. This method of extraction provided a limited volume (amount) of enzyme for supplemental analysis, and a noticeable variation in enzyme activity between samples.

The diagnostic potential of the biochemical test is reinforced by consistent extraction of AChE activity from the synganglion that is greater than background (ΔOD at 30 min 0.006 ± 0.0005 , replicates $n = 84$). Mistaken classification of an individual can occur if low AChE activity, which is associated with the RR genotype, is detected and is actually the result of incomplete and less than homogenous extraction of AChE from the synganglion. In Table 2, eight of the sonicated synganglia of the *B. microplus* Munoz strain were found to be homozygous susceptible and two heterozygous resistant. Based upon the same rationale when synganglia were extracted by grinding seven were diagnosed as homozygous susceptible, two heterozygous resistant, and one homozygous resistant. The validity of the diagnosis of one homozygous resistant individual in the susceptible strain is questionable knowing that grinding can result in lower levels of AChE extraction.

Sonication as demonstrated in this study offers a practical improvement in the basic assay by enhancing the homogenous extraction of AChE from the single

synganglion. Additional enzyme allows for diagnosis of OP-insensitivity for the OP of concern and screening for insensitivity to other related OP class acaricides. The sonication method and the microplate assay of AChE activity as presented allows for (1) a measure of enzyme activity where low activity is diagnostic for the RR genotype; (2) definition of the homozygous susceptible, heterozygous resistant, and homozygous resistant genotype in timed inhibition experiments; (3) the calculation of the time to inhibition of (x)% of enzyme activity by regression analysis of the plot of % activity remaining in the presence of inhibitor versus time, however, this value is difficult to predict in the RR genotype because of low uninhibited AChE activity; and (4) the determination of the concentration of inhibitor necessary to inhibit AChE to (x)% inhibition at a fixed time by titration of the inhibitor and subsequent regression analysis of % activity remaining at the fixed time versus the negative \log_{10} of the inhibitor concentration.

Direct application benefits of AChE extraction by sonication and the OP inhibition assay described, include rapid diagnosis of OP-insensitivity from ticks that survive OP treatment at importation ports, and a diagnostic tool for epidemiological studies of the distribution and extent of OP resistance for control programs such as the Cattle Fever Tick Surveillance and Quarantine Program maintained by USDA-APHIS Veterinary Services. These methods should find utility in all regions of the world where ticks are significant vectors of disease and regulators have a need for diagnostic tools in order to successfully control ticks and mitigate or prevent the spread of disease.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Brenda K. Burkett, Chastity Balero, Chris Santos, Gary Earl, Cindy Massengale, and Wayne Ryan of the Knippling-Bushland U.S. Livestock Insects Laboratory; and Ron Davey, Robert Miller, Dave Krska, and Mary Brumley of the USDA-ARS Cattle Fever Tick Research Laboratory for supplying *B. microplus* ticks and technical expertise.

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